



Effects of autophagy inducers on recombinant antibody production in insect cells

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Abstract

Insect cells have recently proven to be an excellent platform for the high-level production of functional recombinant proteins. Autophagy is an important mechanism that promotes cell survival by eliminating damaged organelles and protein aggregates, and it also may influence recombinant protein production. In the present study, we compared the effects that autophagy inducers rapamycin, everolimus, and lithium chloride exert on recombinant lepidopteran insect cells that secrete an engineered antibody molecule. Compared with nontreatment, treatment with either rapamycin or everolimus prolonged cell growth to allow high cell density, improved viability in the declining phase, and then increased the yield of secreted antibodies. These positive effects appeared to be induced via autophagy since autophagosomes were clearly detected, particularly in cells treated with rapamycin or everolimus. Unlike rapamycin, another autophagy inducer, FK506, was ineffective in insect cells. The addition of an appropriate autophagy inducer may be effective in increasing the productivity of recombinant proteins in insect cells.

Keywords Insect cell culture · Recombinant protein production · Autophagy inducer · Rapamycin · Everolimus · Lithium chloride

1 **Introduction**

2
3 In recent decades, various biologics such as therapeutic antibodies have been developed
4 and approved (Walsh 2006; Weiner 2015; Lai et al. 2018). Such biologics are generally
5 produced as recombinant proteins using cell culture technology and recombinant DNA
6 technology. Mammalian cells such as Chinese hamster ovary (CHO) cells and NS0 cells
7 are major host cells for the production of recombinant proteins that are used as biologics
8 (Butler et al. 2012; Kunert et al. 2016), but mammalian cells have disadvantages such as
9 complicated culture operations and high production costs (Chames et al. 2009). Insect
10 cells have recently been used as an excellent platform for the high-level production of
11 functional recombinant proteins (Felberbaum 2015). Compared with mammalian cells,
12 insect cells are easier and more cost-effective to cultivate, and they can be grown to high
13 cell density in suspension with a serum-free medium. Insect cells can also produce
14 significant amounts of recombinant proteins through post-translational processing and
15 modifications that are similar to those performed in mammalian cells (Luckow 1995;
16 Yamaji 2014).

17 Many recent reports have detailed the influences that autophagy exerts on cell growth,
18 cell-cycle progression, and protein synthesis in mammalian cells (Lee and Lee 2012;
19 Pengo et al. 2013). Autophagy is an intracellular catabolic mechanism that involves the
20 degradation and recycling of undesired components in the cytoplasm. Depending on
21 cell type, intracellular metabolic activity, extracellular nutrient supply, and triggering
22 stimuli (Chen et al. 2018), autophagy not only is a cell-survival mechanism, but also
23 promotes cell death via a mechanism that differs from that of apoptosis and necrosis (Das
24 et al. 2012).

25 Various autophagy inducers have been reported for mammalian cells. Rapamycin is

an autophagy inducer that is a mammalian/mechanistic target of rapamycin (mTOR) inhibitor (Blommaert et al. 1995). Rapamycin forms a complex with FK506-binding protein 12 (FKBP12) that is an immunophilin and peptidyl-prolyl cis-trans isomerase, which binds to mTOR to inactivate the mTOR kinase activity (Lee and Lee 2012; Hausch et al. 2013). Everolimus is an analog of rapamycin that works in a similar manner as an mTOR inhibitor with improved pharmaceutical properties. Everolimus also acts as an autophagy inducer (Cerni et al. 2019). Lithium chloride (LiCl) is another autophagy inducer (Ohmuro-Matsuyama et al. 2019) with an action mechanism for autophagy induction that differs from that of rapamycin and everolimus. LiCl uses an mTOR-independent pathway whereby lithium inhibits glycogen synthase kinase-3 β (GSK3 β) (Motoi et al. 2014; Ha et al. 2014).

In a previous study (Ohmuro-Matsuyama et al. 2019), we examined the effects of LiCl on recombinant insect cells that secrete an engineered antibody molecule in a serum-free culture and found that the addition of lithium promoted recombinant antibody production from insect cells probably via the induction of autophagy. In the present study, we compared the effects that autophagy inducers rapamycin, everolimus, and LiCl exert on recombinant insect cells. Treatment with these agents, in particular rapamycin and everolimus, induced autophagy in recombinant insect cells and led to significant increases in the yields of secreted antibodies.

Materials and methods

Insect cell line and culture medium

Stably transformed 1G3 cells that express a single-chain variable fragment fused with the

Fc region of human IgG1 (scFv-Fc) were previously generated from the *Trichoplusia ni* BTI-TN-5B1-4 insect cell line (High Five; Thermo Fisher Scientific, Waltham, MA, USA) (Sonoda et al. 2012). The cells were maintained at 27 °C in a serum-free medium called Express Five SFM (Thermo Fisher Scientific), which is supplemented with 2.6 g/L of L-glutamine (Nacalai Tesque, Kyoto, Japan), 0.87 g/L of G418 (InvivoGen, San Diego, CA, USA), and 10 mg/L of gentamycin sulfate (Thermo Fisher Scientific).

Autophagy inducers

Rapamycin (Santa Cruz Biotechnology, Dallas, TX, USA), everolimus (LC Laboratories, Woburn, MA, USA), and FK506 (tacrolimus; LC Laboratories) were individually dissolved in methanol to a concentration of 10 mM, and stored at –30 °C until use. LiCl (Nacalai Tesque) was dissolved in phosphate-buffered saline (PBS) to a concentration of 1.5 M.

Cell culture with autophagy inducers

1G3 cells in the exponential growth phase were inoculated at a concentration of 2.0×10^5 cells/cm³ into 6-well cell culture plates containing 2 mL of fresh culture medium. Each solution of rapamycin, everolimus, and FK506 was diluted with dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) to a concentration of 200 µM immediately before use. After the 1G3 cells had adhered, rapamycin, everolimus, or FK506 was added to the culture medium for a final concentration of 200 nM. The final concentration of 200 nM for these reagents was predetermined as more effective than either 100 or 400 nM based on comparison of cell density, viability, and scFv-Fc

production (data not shown). LiCl was added to the culture medium at a final concentration of 30 mM (Ohmuro-Matsuyama et al. 2019). DMSO was added to untreated cells as a control for the testing of rapamycin, everolimus, and FK506, while PBS was added for the testing of LiCl. Cell density was determined by microscopically counting the number of cells with a hemocytometer. Cell viability was judged by trypan blue dye exclusion.

Cell staining using a Cyto-ID autophagy detection kit

Cells were inoculated with respective autophagy inducers in glass-based dishes (Matsunami Glass Ind., Kishiwada, Japan). On day 3 after inoculation, a Cyto-ID autophagy detection kit (Enzo Life Sciences, Farmingdale, NY, USA), following the manufacturer's protocol, was used to stain the autophagosomes in cells with a cationic amphiphilic tracer (CAT) dye that selectively labels accumulated autophagic vacuoles. The cells were then observed via an EVOS FL cell imaging system (Thermo Fisher Scientific).

Enzyme-linked immunosorbent assay (ELISA)

Culture supernatants were analyzed via ELISA to evaluate the concentration of scFv-Fc with the antigen-binding activity using peroxidase conjugated goat anti-human IgG-Fc fragment antibody (Bethyl Laboratories, Montgomery, TX, USA), as previously described (Sonoda et al. 2012).

Glucose consumption

Glucose concentration in the culture supernatant was determined using a commercial kit (Glucose CII-test Wako; Fujifilm Wako Pure Chemical, Osaka, Japan) in accordance with the manufacturer's instructions.

Statistical analysis

Statistically significant differences between two groups were determined using the Student's *t*-test. A *p*-value < 0.05 was considered statistically significant. Asterisks indicate a significant difference (* *p* < 0.05; ** *p* < 0.01).

Results

Cell growth and recombinant protein production with autophagy inducers

First, we investigated the effects of rapamycin, everolimus, and LiCl as autophagy inducers on recombinant insect 1G3 cells stably secreting scFv-Fc molecules. 1G3 cells were cultured in a serum-free medium with each autophagy inducer, and the viable cell density and viability were monitored. Treatment with either rapamycin or everolimus prolonged the cell growth, which led to peak density of treated cells that was higher than that of untreated cells (Fig. 1a; *p* < 0.05). Rapamycin and everolimus also promoted the survival of cells and improved the viability in the decline phase (day 8–12), compared with that of nontreatment (Fig. 1b; *p* < 0.05). By contrast, LiCl-treated cells showed viable cell density that was almost identical to that of untreated cells (Fig. 1a).

We also evaluated the effects on glucose consumption and on the scFv-Fc production

in the culture supernatant. The glucose concentration in the culture supernatant of cells treated with each of the autophagy inducers was kept higher than that in the supernatant of untreated cells (Fig. 1c), although the differences were not significant. Moreover, either rapamycin or LiCl treatment led to a considerable increase in the scFv-Fc concentration following day 8 (Fig. 1d). The yields of secreted scFv-Fc were significantly increased by rapamycin treatment compared with that of nontreatment ($p = 0.0098$ on day 10; $p = 0.0063$ on day 12). Concentrations of secreted scFv-Fc were higher from LiCl-treated cells than from cells without an autophagy inducer ($p = 0.024$ on day 8; $p = 0.0065$ on day 10; $p = 0.0098$ on day 12). Particularly on day 12, the scFv-Fc yield from cells treated with rapamycin and LiCl increased by 38.1 and 41.1%, respectively, compared with that from untreated cells.

Treatment with autophagy inducers such as rapamycin and everolimus led to a marked increase in the number of cells containing autophagosomes compared with untreated cells (Fig. 2). These results indicate that the autophagy inducers improved the productivity of scFv-Fc by stimulating the autophagy pathway in 1G3 cells. ◀ Fig. 2

Effects of FK506 on autophagy and antibody production

FK506, tacrolimus, is known as an immunosuppressive drug similar to rapamycin (Cho et al. 2003), and it activates autophagy in mammalian neuronal cells (Nakagaki et al. 2013; Kim et al. 2017). Since FK506 and rapamycin share the binding site of FKBP12 (Cho et al. 2003), FK506 may act pharmaceutically as an antagonist of rapamycin. When 1G3 cells were treated with FK506 alone, the time courses for viable cell density and viability were nearly equivalent to those of untreated cells (Fig. 3a, b), suggesting ◀ Fig. 3 that FK506 did not induce autophagy in insect 1G3 cells. When cells were treated with

rapamycin and FK506 simultaneously, both the cell density and the viability were depressed compared with treatment with rapamycin alone. Also, the scFv-Fc production by cells treated with both rapamycin and FK506 was reduced compared with cells treated with rapamycin alone (Fig. 3c). Autophagosomes were barely visible in the cells treated with both rapamycin and FK506, although appeared markedly in the rapamycin-treated cells (Fig. 4). These results indicate that FK506 might antagonize and suppress the effects of rapamycin as an autophagy inducer. ◀ Fig. 4

Discussion

Autophagy plays an important role in the promotion of cell survival by degrading and recycling of undesired components in the cytoplasm. Recent reports have established the positive effects that autophagy exerts on cellular growth and protein production in mammalian and insect cell cultures (Lee and Lee 2012; Pengo et al. 2013; Ohmuro-Matsuyama et al. 2019). In the present study, we compared the effects that several autophagy inducers exert on growth and scFv-Fc production by recombinant lepidopteran insect 1G3 cells.

By comparison with nontreatment, treatment with rapamycin or everolimus increased the peak cell density and improved the cell viability in the declining phase (Fig. 1a, b), but such effects were not significant by treatment with LiCl. Reportedly, autophagy stimulation via the downregulation of mTOR complex 1 (mTORC1) signaling protected the cells from programmed cell death that included apoptosis (Chen et al. 2018). Since rapamycin and everolimus are mTORC1 inhibitors, they were expected to protect 1G3 cells from apoptosis. LiCl induces autophagy by inhibiting GSK3 β (Ha et al. 2014; Motoi et al. 2014), which is a mechanism of action that differs from that of rapamycin

1 and everolimus. In a previous study, treatment with LiCl promoted both autophagy and
2 apoptosis in 1G3 cells (Ohmuro-Matsuyama et al. 2019). Therefore, treatment with
3 LiCl might have led to a decrease in viability by promoting apoptosis, whereas rapamycin
4 and everolimus inhibited apoptosis (Lee and Lee 2012).

5 Several papers have shown that glucose starvation induces autophagy (Moruno et al.
6 2012; Neufeld 2012; Ha et al. 2015). Hence, glucose usually plays the role of an
7 upstream effector in the autophagy process. In the present study, the glucose
8 consumption rate of cells treated with each autophagy inducer was decreased compared
9 with that of untreated cells (Fig. 1c). Considering the time course for viable cell density
10 (Fig. 1a), the specific glucose consumption rate of cells treated with each autophagy
11 inducer was definitely lower than that of untreated cells. The decrease in the glucose
12 consumption rate probably resulted from the induction of autophagy in treated cells.

13 1G3 cells treated with autophagy inducers had significant increases in scFv-Fc
14 production compared with that of untreated cells following day 8 (Fig. 1d). On day 12,
15 significantly higher concentrations of secreted scFv-Fc were obtained from LiCl-treated
16 cells (41.1%) and rapamycin-treated cells (38.1%) than from untreated cells.
17 Nevertheless, significantly fewer autophagosomes were detected in the LiCl-treated cells
18 than in the rapamycin-treated cells (Fig. 2). The simultaneous addition of 200 nM of
19 rapamycin and 30 mM of LiCl resulted in time courses for cell density and scFv-Fc
20 production that were similar to treatment with 200 nM of rapamycin alone (data not
21 shown). These results suggest that in LiCl-treated cells, in addition to autophagy,
22 another contributing factor might have played a key role in increasing the scFv-Fc yield
23 and that rapamycin treatment could be more effective than LiCl treatment. The effects
24 of LiCl may vary depending on cell type. In the present study, LiCl was added to 1G3
25 cells at a final concentration of 30 mM based on the findings of a previous study

(Ohmuro-Matsuyama et al. 2019). Ha et al. (2014) reported that CHO cells treated with 10 mM LiCl produced the highest concentration of an Fc-fusion protein, while CHO cells treated with 20 mM LiCl did not grow and cell viability decreased rapidly. Mao et al. (2001) reported that lithium can affect cell proliferation either positively or negatively depending on cell type. Kim et al. (2016) also showed that the suitable concentration of LiCl for antibody production differed among cell types.

FK506, tacrolimus, is an immunosuppressive drug like rapamycin, and it shows antineurodegenerative activities via the process of autophagy in neuronal cells. When treated with up to 30 μ M FK506, prion-infected neuroblastoma cells activate autophagy and express autophagy-related proteins that degrade the abnormal form of prion proteins (Nakagaki et al. 2013). Kim et al. (2017) reported that FK506 induced autophagy in a manner similar to rapamycin in neuroblastoma cells. In addition, FK506 and rapamycin are believed to share a binding site of FKBP12 (Cho et al. 2003). In the present study, 1G3 cells treated with FK506 showed time courses for cell density and viability similar to those of untreated cells (Fig. 3a, b). Treatment with rapamycin alone promoted the survival of 1G3 cells and improved viability in the declining phase compared with nontreatment. However, both the density and viability of cells treated with rapamycin and FK506 together were suppressed compared with cells treated with rapamycin alone (Fig. 3a, b). No autophagosomes were detected in 1G3 cells treated with rapamycin and FK506, even though these appeared markedly in rapamycin-treated cells (Fig. 4). These results indicate that FK506 may inhibit autophagy caused by rapamycin whereas it does not activate autophagy in lepidopteran insect cells. Ding et al. (2018) also found that autophagosomes were invisible in FK506-treated mammalian cells. Effects of the simultaneous addition of everolimus and FK506 on 1G3 cells remain to be investigated.

In conclusion, treatment with rapamycin and everolimus increased the peak cell

density and promoted the survival of cells in the declining phase probably due to the induction of autophagy, which resulted in an enhanced production of recombinant antibody molecules by insect cells. Treatment with a suitable autophagy inducer is a simple and effective approach to enhancing the productivity of recombinant proteins in insect cells.

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Figure legends

Fig. 1 Effects of autophagy inducers on recombinant insect 1G3 cells. Time courses for viable cell density (**a**), viability (**b**), glucose concentration (**c**), and scFv-Fc concentration in the culture supernatant (**d**) in static culture. The scFv-Fc concentration was measured by enzyme-linked immunosorbent assay (ELISA). The Student's *t*-test was used to determine statistically significant differences between autophagy inducer-treated and untreated cells. Asterisks indicate a significant difference (* $p < 0.05$; ** $p < 0.01$). The results represent the means $\pm$ S.D. obtained from three independent experiments. Closed circles, untreated cells; squares, cells treated with 200 nM rapamycin; triangles, cells treated 30 mM LiCl; diamonds, cells treated with 200 nM everolimus

Fig. 2 Staining of autophagosomes in 1G3 cells treated with autophagy inducers on day 3. Autophagosomes were stained using a Cyto-ID autophagy detection kit. Yellow arrows show typical stained autophagosomes in the LiCl-treated cells. White bars indicate 50 μ m

Fig. 3 Effects of rapamycin and FK506 on 1G3 cells. Time courses for viable cell density (**a**), viability (**b**), and scFv-Fc concentration in the culture supernatant measured by ELISA (**c**) in static culture. The Student's *t*-test was used to determine statistically significant differences between treated and untreated cells. Asterisks indicate a significant difference (* $p < 0.05$; ** $p < 0.01$). The results represent the means $\pm$ S.D. obtained from three independent experiments. Closed circles, untreated cells; squares, cells treated with 200 nM rapamycin; triangles, cells treated 200 nM rapamycin and 200 nM FK506; diamonds, cells treated with 200 nM FK506

1

2 **Fig. 4** Staining of autophagosomes in 1G3 cells treated with rapamycin and FK506 on
3 day 3. Autophagosomes were stained using a Cyto-ID autophagy detection kit. White
4 bars indicate 50 μm

5

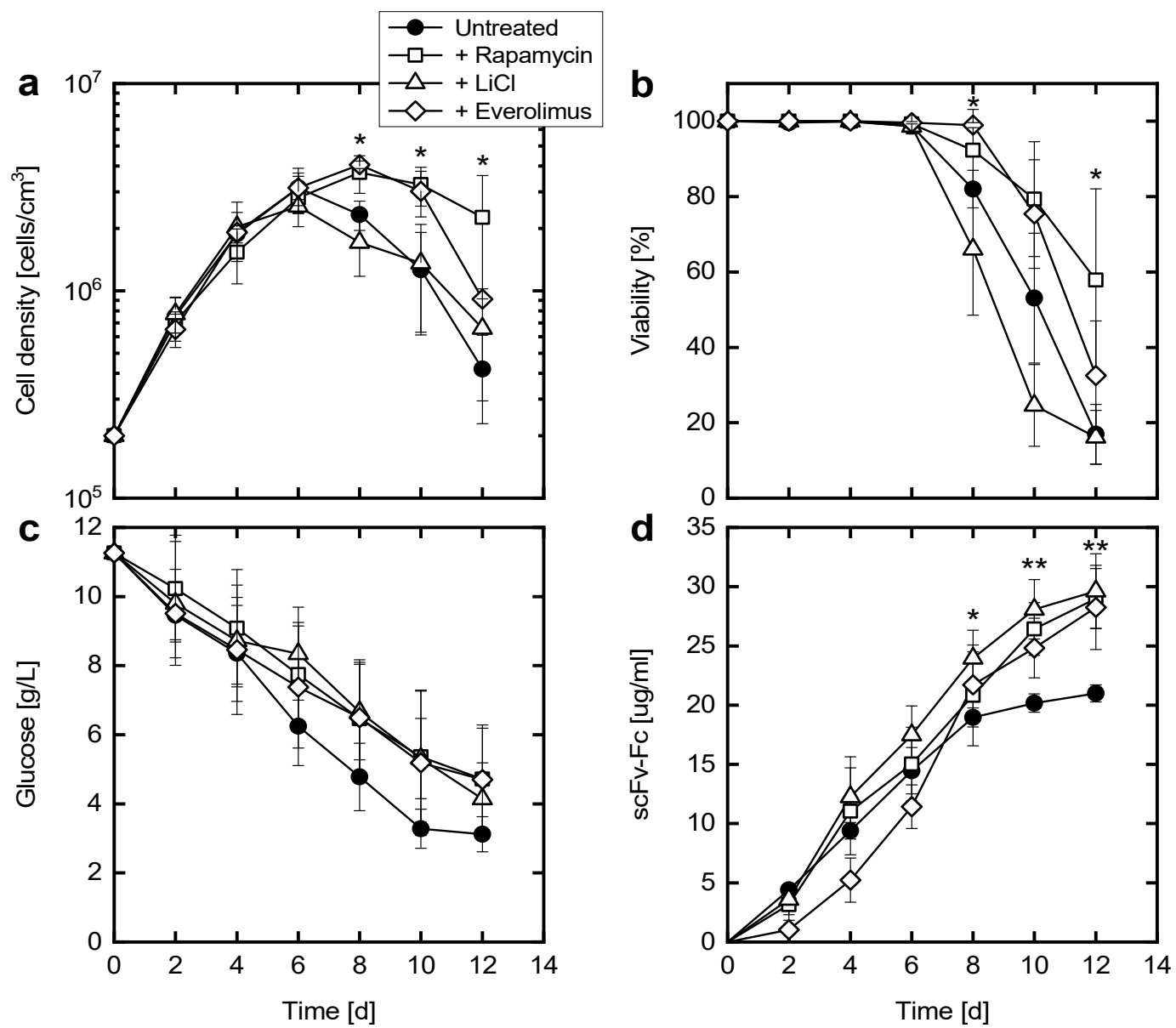


Fig. 1

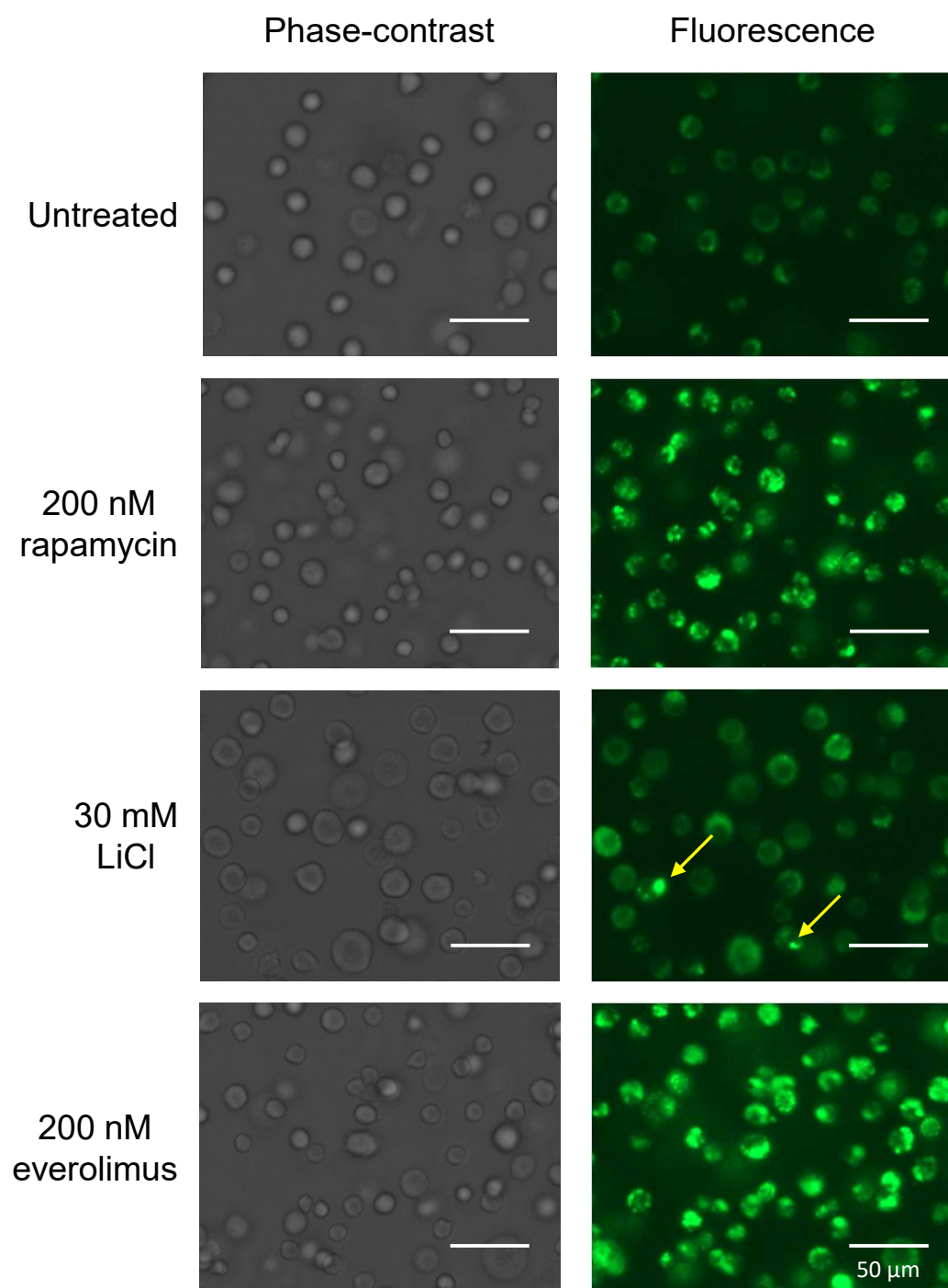


Fig. 2

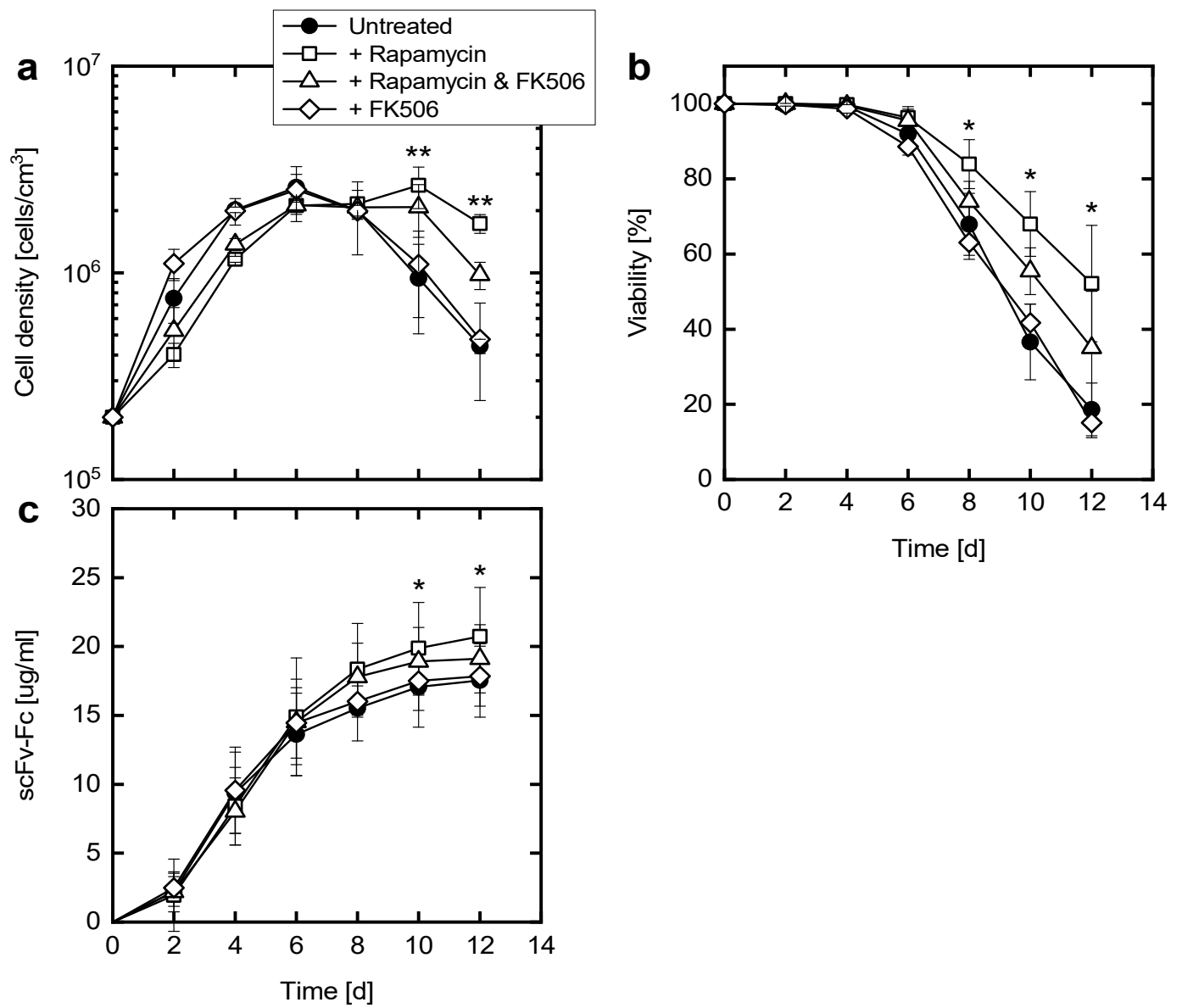


Fig. 3

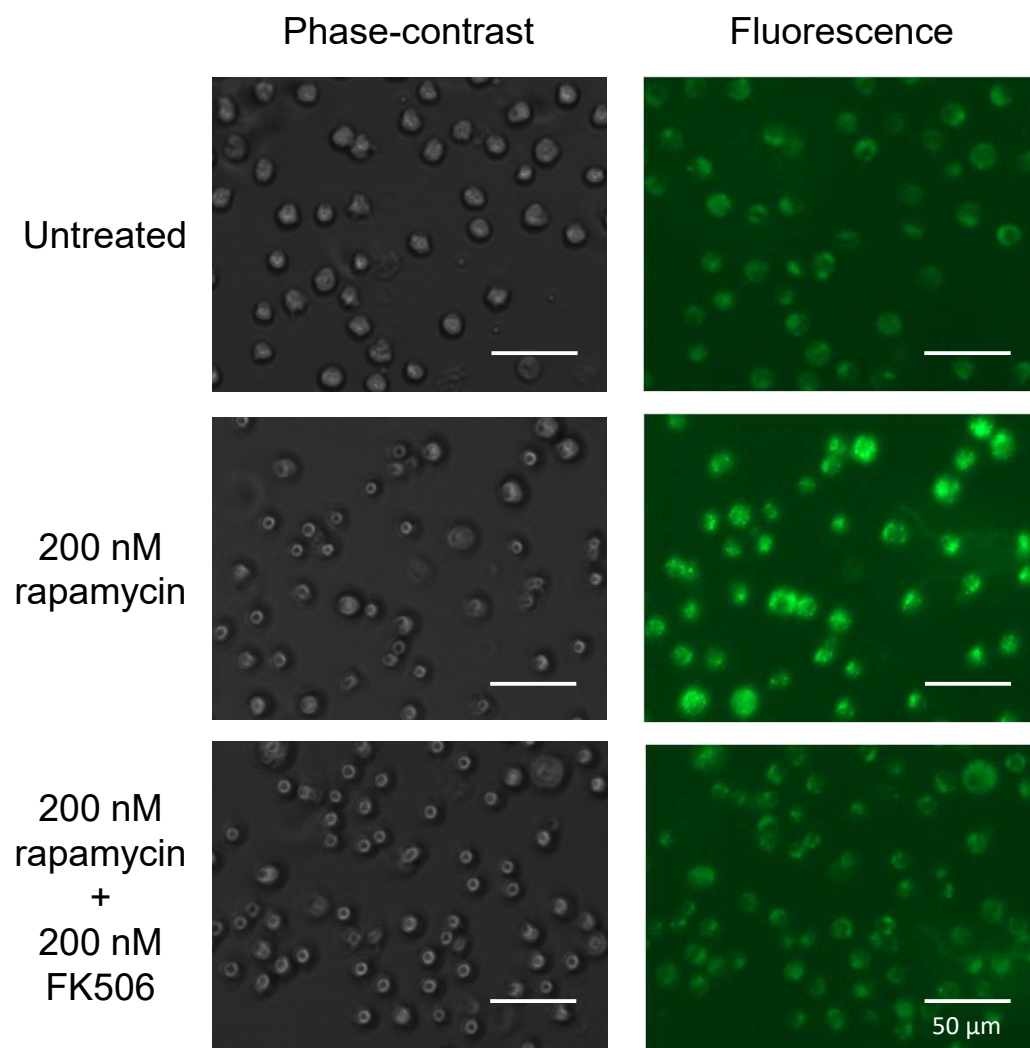


Fig. 4